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van der Zee, E.A.; Naber, P.A.; Disterhoft, J.F.

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AGE-DEPENDENT CHANGES IN THE IMMUNOREACTIVITY FOR NEUROFILAMENTS IN RABBIT HIPPOCAMPUS

E. A. VAN DER ZEE,* P. A. NABER and J. F. DISTERHOFT

Department of Cell and Molecular Biology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611, U.S.A.

Abstract—The distribution of the three subunits of neurofilaments was examined in the hippocampus of young adult rabbits (three months of age), employing a panel of six monoclonal antibodies. Thereafter, age-dependent and subunit-selective changes in neurofilament immunoreactivity in the ageing rabbit hippocampus were studied, using animals of one, three, six, 12, 24, 30, 36, 48, and 60 months. Principal cells, interneurons, axons, and various fibre systems were immunoreactive for all three subunits, although the localization and staining intensity of neurofilament immunoreactivity depended on the antibody used. Small cells immunopositive for the low subunit of neurofilament (presumably glial cells) were found abundantly in the hippocampal formation at one month, and (occasionally) at 30–36 months. Young rabbits (one to three months of age) had high numbers of interneurons stained for the high subunit of neurofilament in the stratum oriens/pyramidal. The number declined and plateaued to approximately 78% at six to 30 months, and further declined and plateaued to approximately 56% at 36–60 months. The first decline may reflect a process of maturation, while the latter decline most likely relates to ageing. Ageing pyramidal cells in 48–60 months animals revealed a slight increase for the low subunit of neurofilament, but no changes for the other subunits. Transient changes in neurofilament immunoreactivity were a striking observation in dentate gyrus granule cells during ageing. The staining intensity for the low subunit of neurofilament decreased gradually from one to 24–30 months until it was no longer detectable in these cells. The immunoreactivity then reappeared, most notably in granule cells lining the hilus, at the age of 36–48 months. By 60 months all granule cells were nearly immunonegative for this subunit. Axonal aberrations, immunoreactive for all three subunits, were found throughout the hippocampal formation. These aberrations first appeared in 24-month-old animals and increased in number and maximal size in older rabbits.

The alterations in neurofilament immunoreactivity in the ageing hippocampus correlated with age-associated learning disabilities in the acquisition of a hippocampally-dependent learning task. The potential relevance of changes in the cytoskeletal profile of hippocampal neurons to age-associated learning and memory disabilities is discussed. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: ageing, axonal aberration, distribution, brain.

The neurofilament (NF) protein triplet is a cytoskeletal constituent, and consists of the low (NF-L), medium (NF-M), and high (NF-H) subunits of apparent molecular weights of 67–69,000, 145–160,000 and 200,000, respectively.^{32–34,48} NF-L can form neurofilaments on its own, whereas NF-M and NF-H can only form heteropolymers with NF-L.²⁹ Destruction and construction of the NF-protein triplet occur continuously in normally functioning neurons,^{7,23} indicating dynamic cellular processes underlying neuronal stability. The neurofilaments are separate

gene products,⁸ which are immunocytochemically distinct.⁹ In neurodegenerative disorders such as Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis as well as in normal ageing, antibodies raised against neurofilaments were used to localize vulnerable neuronal populations immunocytochemically.^{19,39,62,68} Most (but not all) populations of neurons express NF,⁶⁷ and neurofilament is considered a neuronal marker absent in glia.⁶¹ Neurofilaments are thought to serve primarily a structural role, but also play a fundamental role in axonal transport.^{21,25} It is generally accepted that a principal role of neurofilaments is to maintain the calibre of large myelinated axons (see Ref. 44 for review, but see Ref. 67). The phosphorylated forms of NF-M and NF-H account for the structural stability of the mature neuron by their interaction with other cytoskeletal proteins and by protection against proteolysis.^{8,20} Ageing-related changes in cell morphology are most likely preceded by cytoskeletal changes in the perikarya and axons, and disruption

*To whom correspondence should be addressed.

Abbreviations: ChAT, choline acetyltransferase; DAB, diaminobenzidine; DG, dentate gyrus; EDTA, ethylenediaminetetra-acetate; EGTA, ethyleneglycol-bis(aminoethylether)tetra-acetate; ETOH, ethanol; HRP, horseradish peroxidase; IR, immunoreactivity; NF, neurofilament; NF-H, high molecular weight subunit of NF; NF-L, low molecular weight subunit of NF; NF-M, medium molecular weight subunit of NF; NSS, normal sheep serum; PBS, phosphate buffered saline; PmSF, phenylmethylsulfonyl fluoride.

of structural proteins will lead to cell disintegration.⁷⁰ Therefore, changes in the immunoreactivity (IR) for neurofilament may indicate significant alterations in normal cell functioning.

The hippocampus is known to be critically involved in mnemonic processes, and this brain region is especially affected by ageing and in diseases associated with ageing.^{1,65} Besides rats, the rabbit has been widely used in behavioural and biological studies of ageing.^{57,58,72,73} Trace eyeblink conditioning is a hippocampally-dependent learning task useful to study behavioural, neurochemical, neurophysiological and biophysical aspects of associative learning in the young and aged hippocampus.^{11,40,41,59,71}

The rabbit eyeblink conditioning model has been promoted for its direct behavioural parallels with studies in ageing humans.^{55,57,72} In rabbits, a reduced acquisition rate and an increase in the proportion of animals failing to learn this task were observed with advancing age.⁶⁰ However, less is known about possible neurochemical correlates that may occur in the ageing rabbit hippocampus. Previously, we described alterations in calcium binding proteins in the ageing rabbit hippocampus.¹⁰ In the present study, we seek cytoskeletal alterations which may correlate with the behavioural impairment of ageing rabbits. Plastic changes in the structure of neuronal processes occur in relation to enhanced neuronal activity induced by learning and memory or long-term potentiation.^{17,22} Alterations in the cytoskeletal profile of ageing neurons may interfere with the capability of such structural changes to take place, and are likely to contribute to age-associated disabilities in learning and memory. Cytoskeletal alterations may be detected employing a battery of anti-NF antibodies. However, no detailed description of the distribution of the different NF-subunits in the rabbit hippocampus is available, neither for young adults nor for the ageing subjects. Proper organization of neurofilaments requires a certain ratio of the different NF-subunits.³⁷ Therefore, we first describe the distribution of NF-L, NF-M, and NF-H in the young adult rabbit hippocampus. Thereafter, we examine and quantitate naturally occurring changes in the hippocampal NF-content with advancing age. Part of this study has been published in abstract form elsewhere.⁴⁶

EXPERIMENTAL PROCEDURES

Animals

Forty-seven female New Zealand White rabbits (HRP Inc., Denver, PA) of nine different ages were used in this study. The age groups and *n*/group were 1(5), 3(6), 6(5), 12(5), 24(7), 30(7), 36(6), 48(4), and 60(2) months-of-age. The animals were housed individually, with food and water available *ad libitum*.

Immunocytochemical procedure

Rabbits were deeply anaesthetized with a combination of ketamine (0.35 ml+0.44 ml/kg) and xylazine (0.1 ml+0.066 ml/kg). Fixation of the brain was carried out by

transcardial perfusion of 700 ml of a fixative consisting of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer at pH 7.4. Fixation was preceded by a prerinse with 100 ml saline solution at a perfusion speed of 25 ml/min. The brains were removed, stored overnight in 30% buffered sucrose at 4°C for cryoprotection, and coronally sectioned on a cryostat microtome at a thickness of 20 µm.

Six different monoclonal antibodies raised against neurofilaments were used; two for each molecular weight subunit. For NF-L, NR4 (Sigma, anti-68000 mol. wt raised against pig spinal cord NF-L) and Mab1615 (Chemicon, anti-70,000 mol. wt raised against enzymatically dephosphorylated pig NF-L) were used, both at 1:500. For NF-M, Mab1621 (Chemicon, anti-145000 mol. wt raised against rat NF-M) and NN18 (Sigma, anti-160,000 mol. wt raised against pig spinal cord NF-M) were used at 1:50 and 1:500, respectively. For NF-H, N52 (Sigma, raised against enzymatically dephosphorylated pig NF-H) and Mab1623 (Chemicon, raised against rat NF-H) were used at 1:500 and 1:50, respectively. Brain sections of young and aged rabbits were paired during the immunoprocessing to minimize variability induced by the different incubation steps. Prior to the primary and secondary antibody steps, the sections were preincubated in 5% normal sheep serum (NSS; Amersham) in phosphate-buffered saline (PBS) for 30 min. The sections were then exposed to one of the primary antibodies with the addition of 1% NSS overnight at room temperature under gentle movement of the incubation medium. After rinsing in PBS, the sections were incubated for 2 h at room temperature in biotinylated sheep anti-mouse IgG, F(ab')₂ fragment (Amersham; 1:200). The sections were rinsed in PBS and incubated in Streptavidin-biotinylated horseradish peroxidase (HRP) complex (Amersham; 1:200) for 2 h at room temperature. During all incubation steps 0.5% Triton X-100 was added. Thereafter, the sections were thoroughly rinsed in PBS and Tris buffer, and finally processed with the diaminobenzidine (DAB)-H₂O₂ reaction (30 mg DAB and 0.01% H₂O₂/100 ml Tris buffer), guided by a visual check. The immunocytochemical procedure for each antibody was executed simultaneously on brain sections from all animals to equalize all incubation steps in order to minimize artificial variation in staining intensity. The sections were mounted and coverslipped for light microscopic inspection.

Near adjacent brain sections stained for NF-M and NF-H of six aged animals [60 (*n*=2), 48 (*n*=2), and 36 (*n*=2) months) with the highest incidence of axonal aberrations (see Results) were stained for somatostatin and choline acetyltransferase (ChAT). Somatostatin was visualized by means of the polyclonal rabbit antibodies S309 (1:6000) or S320 (1:6000), respectively directed against the first 14 (S309) and last 12 (S320) amino acids of somatostatin-28. The anti-somatostatin antibodies were kindly donated by Dr Benoit.^{3,38} The polyclonal goat anti-ChAT antibody (1:1000) was kindly donated by Dr L.B. Hersh.⁵ After overnight incubation at room temperature, the sections were subsequently incubated in biotinylated goat anti-rabbit IgG (1:100, Zymed) or biotinylated rabbit anti-goat IgG (1:100, Zymed) for somatostatin and ChAT, respectively. The immuno- and DAB-processing hereafter was identical to that described above for neurofilaments from the secondary incubation step on. During all incubation steps for somatostatin and ChAT 0.5% Triton X-100 was added to the medium.

Standard control experiments were performed by omitting the primary antibody step, or by replacing the primary antibody by normal serum. All these control experiments yielded immunonegative results.

Phosphorylation-dependent immunoreactivity

In order to determine whether phosphorylation interfered with the immunoreactivity for the NF antibodies employed

in the ageing study, we examined the effect of dephosphorylation on the immunocytochemical results obtained with antibodies Mab1615, NN18, and N52, raised against NF-L, NF-M, and NF-H, respectively. Fixed brain sections of young adult rabbits (three months) were incubated with 400 µg/ml bovine intestinal mucosa alkaline phosphatase in 0.1 M Tris-HCl (pH 8.0) at room temperature for 5 h, a slight modification of the method described by Siegel *et al.*⁵⁴ N52 and NN18 served as controls in this experiment because it is known that N52 and NN18 are insensitive to the state of phosphorylation of their epitopes.⁵² In addition, a blot (see below) was preincubated with 200 µg/ml bovine intestinal mucosa alkaline phosphatase in 0.1 M Tris-HCl (pH 8.0) at 37°C for 1 h before being probed with Mab1615.

Western blot procedure

Animals of three months-of-age were deeply anaesthetized and decapitated. The brain was quickly removed from the skull, put on ice and the hippocampi were rapidly dissected within 1.5–2 min. Blocks of the middle portion of the hippocampus (containing all subregions) were sampled and homogenized in a protease phosphatase buffer containing (in mM) 80 β-glycerophosphate, 10 KPO₄, 1 EDTA, 5 EGTA, 10 MgCl₂, 2 dithiothreitol, 1 Na-ortho-vanadate, and 100 µg/ml Pepstatin A, 10 µg/ml Leupeptin, and 40 µg/ml PMSF, pH 7.25. The buffer contained 10% ETOH. The tissue was then centrifuged at 105,000 g for 70 min in a Beckman ultracentrifuge (model L8-70). Protein concentrations were determined according to Lowry *et al.*,³⁶ with bovine serum albumin as a standard. Proteins were separated on sodium dodecyl sulphate-polyacrylamide (8.5%) gels and then transferred to Nytran membrane (Schleicher and Schuell) as described by Burnette.⁶ The blots were probed with anti-NF antibodies overnight at 4°C. In addition to the above mentioned antibodies, a well characterized polyclonal antibody raised against the N-terminal head of NF-L 28 served as a control antibody for the two other NF-L antibodies. [¹²⁵I]Protein A was used to detect antigen-antibody complexes.

Analysis

All analyses were performed on four coronal sections/animal at the dorsal hippocampus corresponding to level 53–63 according to the rabbit brain atlas of Shek *et al.*⁵³ The number of NF-L and NF-H-positive interneurons was counted in the CA1–CA3 strata oriens and pyramidale of four sections/animal. Cells were only counted when the nucleus was in the plane of section. The length of the stratum oriens was measured, and the total number of cells/animal was calculated/1.0 mm. The presence of NF-L, NF-M, and NF-H throughout the hippocampal formation was determined in a semiquantitative way in young adult (three months) rabbits with values ranking high (+++), medium (++), moderate (+), low (±), or none (–). The staining-intensity for the CA1 and CA3 pyramidal cells could not be measured by image analysis due to the (variable) presence of NF-positive axons traversing and terminating within the pyramidal cell layer. The NF-content of the pyramidal cells was therefore semi quantified, with high (4), medium (3), moderate (2), low (1), and none (0) as values.

Two complementary measures were determined for the granule cells of the dentate gyrus (DG). 1) The number of granule cells immunoreactive for NF-L were counted; starting at the beginning of the inner blade, a line of an ocular grid was placed perpendicular on the granule cell layer at intervals of 100 µm. The number of immunonegative and immunopositive granule cells crossed by the line was determined. 2) The length of the granule cell layer populated with immunopositive granule cells was determined by examining the inner rows of the granule cell layer. The total length of the granule cell layer was set at 100%, and any area larger than 20 µm (corresponding to approximately three adjacent

granule cells) devoid of immunopositive granule cells was subtracted from the total length.

The number of axonal aberrations were counted in the stratum oriens, stratum lacunosum moleculare and the stratum moleculare of the DG in four sections/animal for all ages. The surface area of the analysed regions was estimated, and the number of axonal aberrations was expressed/mm². An axonal deformation was counted as an aberration when it was at least 4 µm in diameter. The characteristic shape and size as well as the often cluster-like appearance of the axonal aberrations made it possible to differentiate them from naturally occurring axonal swellings.

Statistics

Analysis of variance was used to test for significant age effects with a probability level of $P < 0.05$ used as an index of statistical significance.

RESULTS

Western blot analysis

Western blot analyses for all but one of the NF antibodies revealed that the immunoreactive bands in rabbit hippocampal tissue were found at the expected migration positions (Fig. 1). The monoclonal antibody Mab1615 (lane 3) seemed to recognize both the 68,000 and 70,000 mol. wt NF-L, recognized by NR4 (lane 1) and the anti-N-terminal NF-L antibody (lane 2), respectively. The NF-M antibody NN18 (lane 5) showed some minor cross-reactivity with NF-H, whereas the NF-H antibody N52 (lane 6) displayed some minor cross-reactivity with NF-M (160,000 mol. wt). No additional bands appeared with the antibodies used for immunocytochemistry (lanes 1, 3, 4, 5 and 6) which would be indicative of a specific cross-reactivity with unknown epitopes. In our hands, however, the NF-H antibody Mab1623 did not show an immunoreactive band in the western blot analyses, although it showed clear immunostaining in aldehyde-fixed brain tissue (Table 1).

Distribution of neurofilament immunoreactivity in the young adult hippocampus

The distribution of the three different neurofilament molecular weight subunits was studied in 3-month-old animals using a panel of six monoclonal antibodies (Table 1). NF-IR was present in the cell soma, dendrites and axons, with the nucleus notably devoid of immunoreactivity. The principal cells and interneurons were immunoreactive for all types of subunits, whereas glial cells were always immunonegative at this age. However, the localization and staining intensity of NF-IR depended on the antibody used (Table 1). In general, the CA3/CA4 pyramidal cells were more densely stained for neurofilaments than the CA1/CA2 pyramidal cells (Tables 1, 2). The labelling in the pyramidal cells was most prominent in the soma, and considerably less so in the basal and apical dendrites. Dendritic staining for all three subunits (most notably seen in the trunk) was more clear in interneurons.

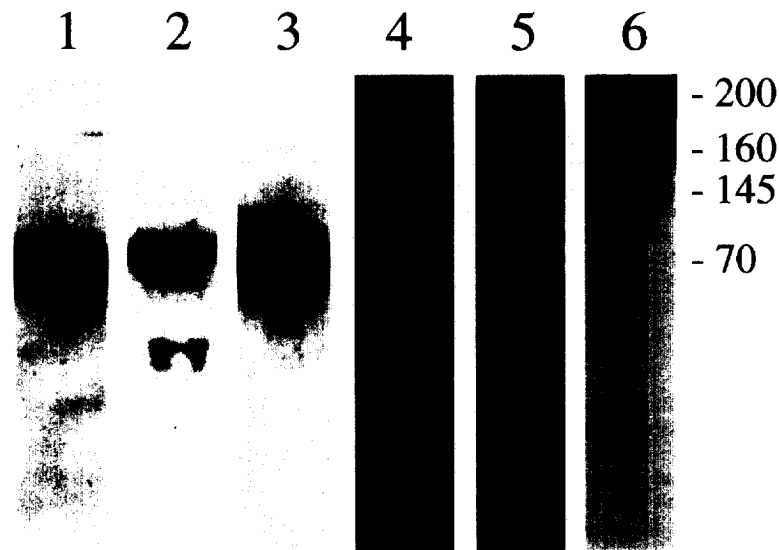


Fig. 1. Antibody specificity as revealed by western blotting. Immunoreactive bands in rabbit hippocampal tissue were found at the expected migration positions. Lane 1: NR4 (68,000 mol. wt); lane 2: anti N-terminal NF-L (70,000 mol. wt); lane 3: Mab1615 (70,000 mol. wt); lane 4: Mab1621 (145,000 mol. wt); lane 5: NN18 (160,000 mol. wt); lane 6: N52 (200,000 mol. wt). The molecular weight is indicated at the right.

Table 1. Distribution of neurofilament-immunoreactive neurons and fibres in the young adult hippocampus of the rabbit

Hippocampal region	68,000 NR4	70,000 Mab1615	145,000 Mab1621	160,000 NN18	200,000 N52	200,000 Mab1623
Principal cells						
subiculum	-/+	++	+	-	++	+
pyramidal cells CA1	-/+	+	+	-/+	+	+
pyramidal cells CA2	-/+	+	+	-/+	+	+
pyramidal cells CA3	-/+	++	++	-/+	+	++
pyramidal cells CA4	-/+	++	++	-/+	+	++
granule cells	-/+	+++	-/+	-/+	-/+	+
Interneurons						
stratum oriens	-	++	+	-	++	+
stratum pyramid.	-	+	+	-	++	-
stratum radiatum	-	-/+	+	-	+	+
hilar region	-	-/+	+	-	+	+
Axons						
subiculum	-/+	++	++	++	++	-
alveus	+++	++	++	+	+	++
stratum oriens	++	++	++	++	++	+
stratum radiatum	+	++	++	+	++	-/+
perforant path	+++	++	++	+	++	+
mol. layer DG	++	++	++	++	++	-
mossy fibres	-	+++	+++	+++	+	-
fimbria	++	++	++	++	+	+

The presence of immunoreactivity was rated as absent (-), faint (-/+), moderate (+), strong (++) or intense (+++).

However, the most striking differences between the antibodies we used became apparent in this cell group (Table 1). For example, no labelling for NF-L and NF-M was found in interneurons employing the 68,000 (NR4) and 160,000 (NN18) mol. wt antibodies, whereas the labelling for NF-L and NF-M evaluated with the 70,000 (Mab1615) and 145,000 (mab1621) mol. wt antibodies revealed clear staining. Granule cells were stained most prominently with the 70,000 mol. wt antibody (Table 1, Fig. 4B). In addition to cell bodies and dendrites,

diverse fibre systems were intensely immunoreactive. The alveus and fimbria stained for all NF-subunits (Table 1). The mossy fibre system was heavily stained for the 70,000, 145,000 and 160,000 mol. wt antibodies. However, there was no clear relation between the staining intensity of the granule cells and that of their associated mossy fibres for the respective antibodies. Besides these densely stained fibre systems, axons immunopositive for all NF-antibodies were found throughout the hippocampal formation.

Table 2. Semiquantified staining intensity (\pm S.E.M.) of pyramidal cells in CA1 and CA3 for neurofilament-L (70,000 mol. wt) and neurofilament-H (200,000 mol. wt)

Age in months	70,000 CA1	70,000 CA3	200,000 CA1	200,000 CA3
1 (5)	1.8 (\pm 0.54)	2.3 (\pm 0.17)	1.8 (\pm 0.20)	2.2 (\pm 0.18)
3 (6)	2.0 (\pm 0.15)	3.0 (\pm 0.28)	1.6 (\pm 0.12)	2.3 (\pm 0.16)
6 (5)	2.2 (\pm 0.55)	3.2 (\pm 0.41)	1.7 (\pm 0.12)	2.7 (\pm 0.23)
12 (5)	2.0 (\pm 0.36)	3.2 (\pm 0.52)	1.8 (\pm 0.23)	2.4 (\pm 0.19)
24 (7)	2.3 (\pm 0.55)	2.6 (\pm 0.42)	2.2 (\pm 0.17)	2.3 (\pm 0.13)
30 (7)	2.3 (\pm 0.22)	2.8 (\pm 0.42)	2.0 (\pm 0.16)	2.0 (\pm 0.14)
36 (6)	1.8 (\pm 0.30)	2.5 (\pm 0.22)	1.9 (\pm 0.13)	2.3 (\pm 0.12)
48 (4)	2.8 (\pm 0.54)	3.0 (\pm 0.46)	1.8 (\pm 0.19)	2.5 (\pm 0.34)
60 (2)	3.5 (\pm 0.48)	2.5 (\pm 0.50)	1.7 (\pm 0.37)	2.8 (\pm 0.37)

No significant differences were found between the different age groups (the number of animals are indicated between parentheses) in pyramidal staining-intensity for NF-L and NF-H, although there is a tendency of increased NF-L-IR in the CA1 pyramidal cells at the ages of 48 and 60 months.

Phosphorylation state of neurofilaments and antibody binding

Age-dependent changes in NF-IR were studied using Mab1615 (NF-L; 70,000 mol. wt), NN18 (NF-M; 160,000 mol. wt), and N52 (NF-H; 200,000 mol. wt). We focused on these monoclonal antibodies to determine age-related changes in NF-IR because of the high quality of the immunostaining obtained with them. First, it was determined whether the epitopes on the rabbit neurofilaments were influenced by dephosphorylation, since the phosphorylation state of neurofilaments may determine the antigenicity to the antibodies used.⁵⁶ Dephosphorylation of fixed brain sections by alkaline phosphatase revealed no changes in the immunoreactivity for NF-M and NF-H, whereas the immunoreactivity for NF-L vanished (data not shown). These results indicate that the epitopes for N52 and NN18 are phosphorylation-independent, while Mab1615 recognized only the phosphorylated form of NF-L in the rabbit brain. This finding was confirmed by the total loss of NF-L-IR in blots preincubated with alkaline phosphatase before antibody probing (data not shown).

Age-dependent changes in neurofilament immunoreactivity: postsynaptic aspects

In one-month-old animals a slightly different staining pattern was found with the 70,000 mol. wt antibody as compared to all other ages (Fig. 2). Small cells (maximum diameter approximately 10 μ m) were found scattered in the alveus and fimbria (Fig. 2D) of all animals, and throughout the hippocampal formation in two out of five animals. Similar cells were found with the antibody raised against N-terminal head of NF-L (data not shown). In these two animals similar cells were observed in the neocortical areas. The cells were irregularly shaped (somewhat more elongated in the fibre tracts and relatively more round in gray matter), showed patches of dense immunoprecipitate in their soma, and generally lacked immunostained processes. Occasionally, these cells were closely associated with interneurons (Fig.

2B). Based on their predominant location in white matter, and their size and shape, these cells most likely represent glial cells. In addition to the observed presence of the presumed glial cells, CA1 pyramidal cells (Fig. 2A) and the hilar interneurons (Fig. 2C) tended to reveal a higher staining intensity in these two animals.

Interneurons in strata oriens, pyramidale, radiatum, and the polymorphic region of the hilus were immunopositive for NF-L and NF-H, but immunonegative for NF-M (NN18) at all ages (data not shown). The interneurons in the strata oriens and pyramidale were most clearly stained for NF-L and NF-H, and could therefore be best quantified for the different ages. Figure 3 depicts the age-dependent number of immunoreactive cells for both markers. Young rabbits (one- to three-months-old) had high numbers of NF-H-positive interneurons. This number declined and plateaued to approximately 78% at the age of six to 30 months, and further declined and plateaued to approximately 56% at the age of 36–60 months. No change in the number of NF-L-positive interneurons occurred during ageing (Fig. 3).

The staining intensity of the CA1 and CA3 pyramidal cells was estimated for NF-L and NF-H (Table 2). Low levels of immunoreactivity were found for NF-M at all ages (data not shown). In general, CA3 pyramidal cells stained darker for neurofilaments than CA1 pyramidal cells, with a higher intensity for the 70,000 mol. wt over the 200,000 mol. wt antibody. No significant changes were found for NF-H with advancing age, whereas a slight increase was found for NF-L at the ages of 48 and 60 months.

No age-related changes were found for NF-M and NF-H in the granule cells, whereas a striking decrease in immunostaining for NF-L became apparent (Figs 4, 5). Two complementary measures were taken to quantify this phenomenon (see Experimental Procedures). In young animals (one month), nearly the entire granule cell layer contained densely stained cells (Figs 2C, 4A, 5), and the vast majority (80.1%, Fig. 5) of the granule cells expressed high levels of

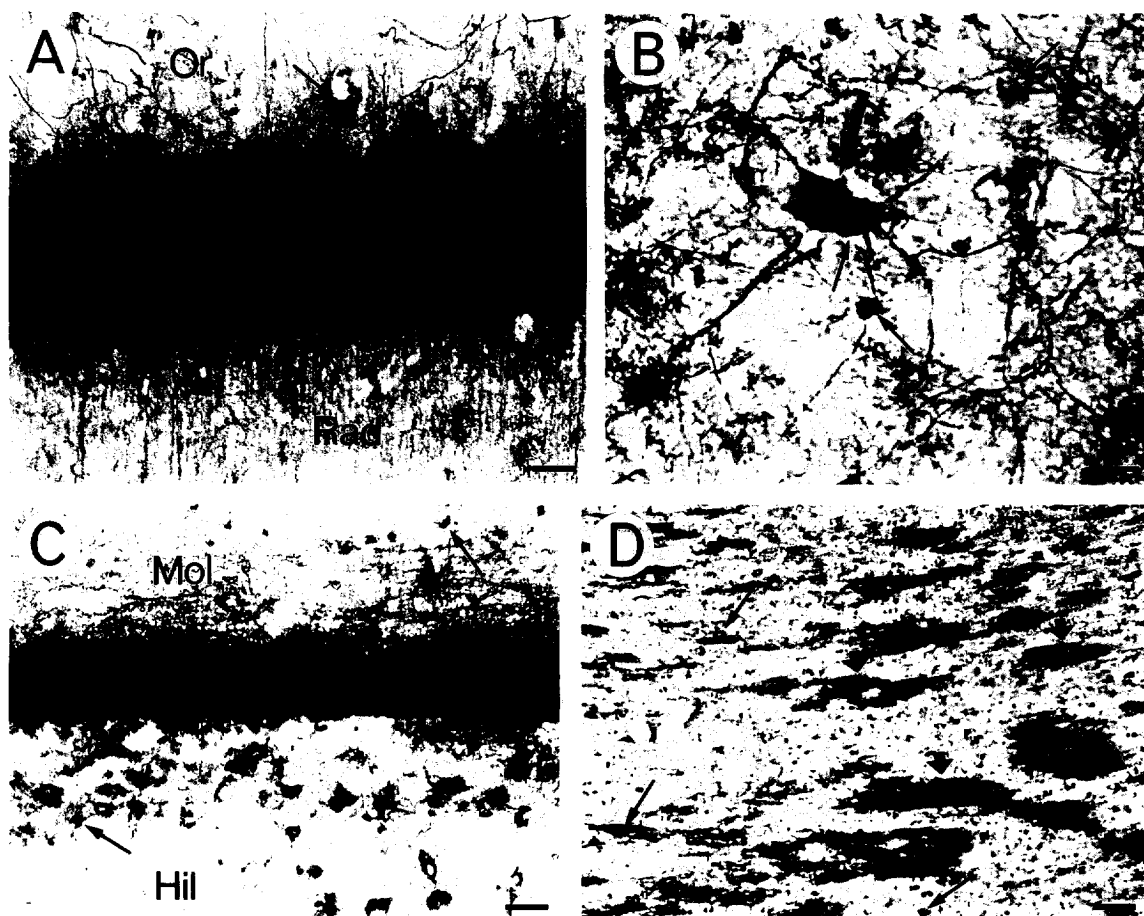


Fig. 2. At the age of one month, pyramidal (A) and granule cells (C) are densely stained for NF-L (Mab1615). In the stratum oriens, stained interneurons (large arrow in B) are encountered, occasionally associated with immunoreactive astrocytes (small arrows in B). Immunoreactive astrocytes (small arrows in A–D) are found throughout the hippocampal formation at this age, including the fimbria (D). Within the fimbria, grouped axons are immunoreactive (small, bold arrows in D). Or, stratum oriens; Rad, stratum radiatum; mol, stratum moleculare; Hil, hilar region. Scale bars in A and C=25 μ m, in B=12 μ m, in D=50 μ m.

NF-L. With advancing age, the number of densely-stained granule cells gradually decreased, in a gradient from the superficial, outer rows of granule cells to the inner rows (Fig. 4), followed by a discontinuous, patchy staining of granule cells, e.g., see the large arrow in Fig. 4D at the age of 12 months. The number of immunoreactive cells decreased from 80.1% (one month) to 5.26–5.98% (24–30 months; Figs 4E, 5). However, at the ages of 36 and 48 months, the number increased significantly ($P < 0.05$) to 17.7 and 18.4%, respectively, resulting in a more continuous staining of the inner rows of the granule cell layer (Fig. 4F, G), occupying approximately 48 and 46% of the granule cell layer. The reappearance of NF-L-IR was not solely restricted to the granule cells. In animals of 30–36 months (three and four animals, respectively) a reappearance of the 70,000 mol. wt-IR was observed in part of the hilar interneurons and some glial cells (Fig. 4F). However, NF-L-IR was absent in the granular cells of the two

60-month animals available (Fig. 4H), whereas other neuronal elements in the DG revealed no consistent changes.

Age-dependent changes in neurofilament immunoreactivity: presynaptic aspects

Axonal aberrations were observed for NF-M stained with NN18 and NF-H stained with N52 (Fig. 6). Spare sections of ageing animals stained for NF-L using NR4 revealed similar axonal aberrations as that found with NN18 and N52, whereas no aberrations were found for NF-L using Mab1615. The axonal aberrations were found throughout the hippocampal formation, but dominated in stratum lacunosum moleculare (Fig. 6B), where approximately 62% of all aberrations were found at the age of 60 months. At the ages of 24–36 months most aberrations were found individually or were clustered in small groups, whereas at the ages of 36–60 months large clusters

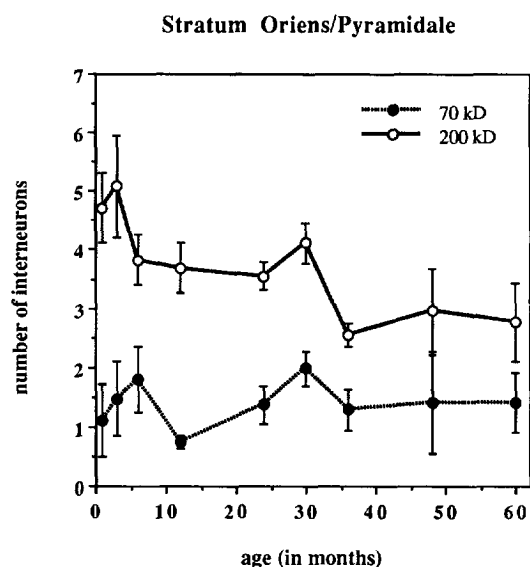


Fig. 3. Numerical density of interneurons immunoreactive to NF-H (open circles and solid line) and NF-L (closed circles and dashed line) /1.0 mm length of the strata oriens/pyramidale during the course of ageing. Bars represent S.E.M. See Table 2 for the number of animals studied/age group.

were frequently encountered, occasionally with more than 100 aberrations. Axonal aberrations could be found in other brain regions, like the cortical areas, as well, although they rarely clustered. The appearance (in number, size and location) of the aberrations for NF-L, NF-M, and NF-H were comparable, and therefore only the NF-H-positive aberrations (which revealed the highest staining intensity) were quantified (Fig. 7). In the age-range studied, the first appearance of the aberrations in considerable numbers was observed in animals of 24 months (Fig. 6A), and increased in number and maximal size (increasing from approximately 2.5 μ m to 12 μ m in animals of 24 and 60 months, respectively) in older animals (Fig. 6B, D). The number of axonal aberrations was counted in the stratum oriens, lacunosum moleculare, and moleculare of the DG, and expressed/mm². Taken together, a gradual increase in the presence of these aberrations was found in the hippocampal formation with advancing age (Fig. 7).

We used antibodies against somatostatin and ChAT to study whether the fibres displaying the NF-positive aberrations could be somatostatinergic or cholinergic. It was demonstrated that the hippocampus of old rats contained ChAT-positive aberrations,¹⁶ and somatostatin-positive aberrations have been observed in the hippocampus of mice (Van der Zee and Compaa, unpublished observations). The ageing rabbit hippocampus revealed decreased immunoreactivity for both somatostatin and ChAT (data not shown). Incidentally, some small (circa 5 μ m in diameter) somatostatin-positive axonal aberrations were found in the ageing hippocampus (at the age of 36 months or older), whereas no

ChAT-positive axonal aberrations were found at any age. Thus the fibres with aberrations did not appear to be cholinergic and at best some could be somatostatinergic.

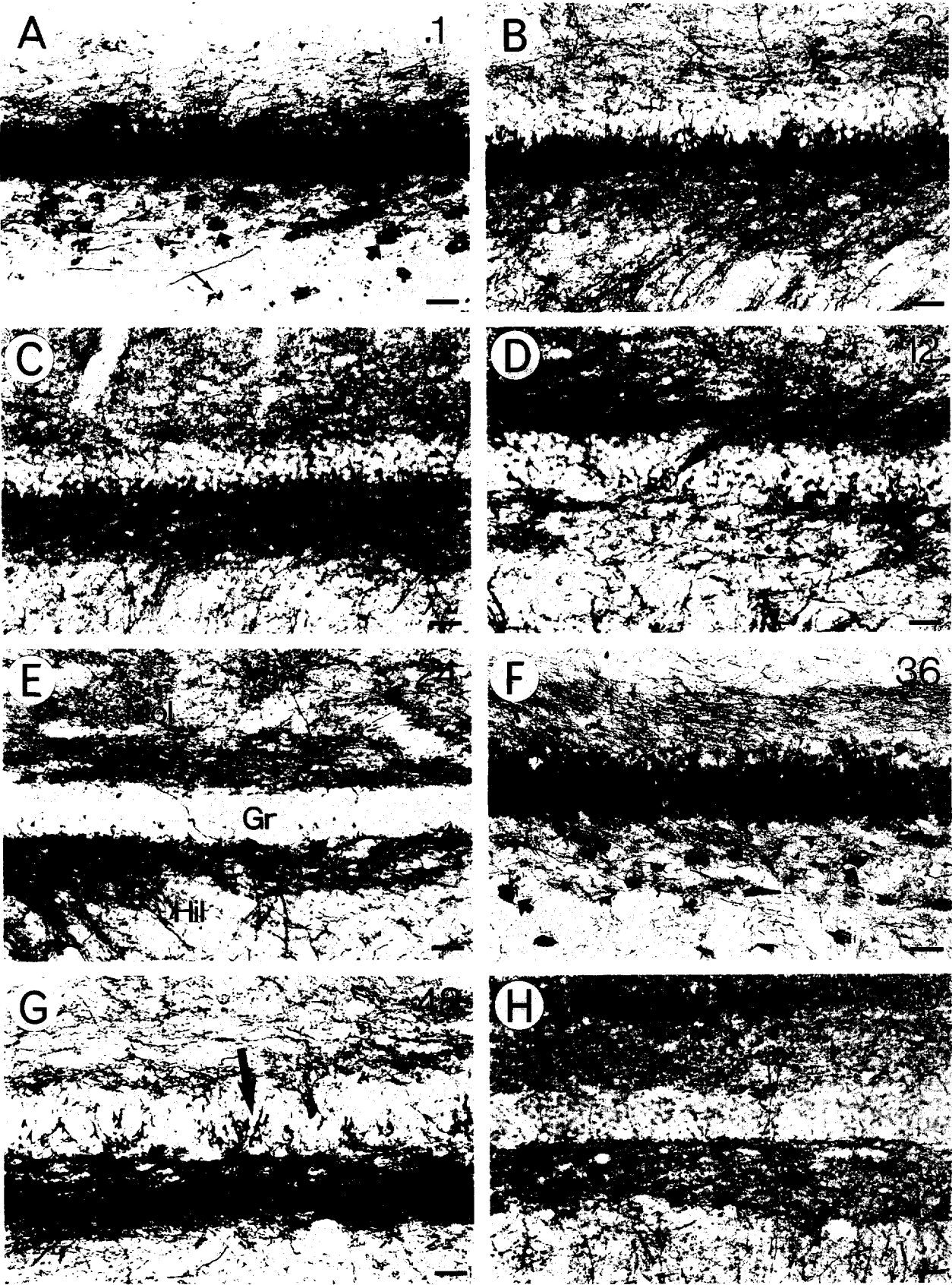
DISCUSSION

Distribution of the three subunits of neurofilament in the young adult rabbit hippocampus

Recognition of rabbit neurofilaments by the three antibodies used for all ages studied (Mabs 1615, NN18, and N52) was demonstrated with western blots of hippocampal tissue, which revealed clear immunoreactive bands at the expected migration positions. These results support the specificity of the obtained immunocytochemical data discussed below.

The distribution pattern of NF-IR depended somewhat on the molecular weight subunit studied. For each subunit, however, the immunoreactivity in the rabbit hippocampus predominated in CA3/CA4 pyramidal cells; in interneurons of the strata oriens, strata pyramidale, and the hilar region; in fibres of the alveus and hippocampus generally; and in principal cells and fibres of the subiculum. CA1 pyramidal cells and DG granule cells were moderately stained. This distribution pattern resembled patterns described for the hippocampus of the guinea-pig,⁶⁷ rat,^{50,74} gerbil,^{14,42} rhesus monkey,⁵⁴ and human.^{27,69} There were some characteristic species differences, though. For example, NR4 displayed densely-stained interneurons in rat⁷⁴ but not in rabbit. This difference may relate to differences in immunological features of neurofilaments between mammals.⁵¹ However, different antibodies were used in most of the above mentioned studies from those employed here, and our results and those from previous work show that the observed distribution pattern for neurofilaments depends on the antibody used, even within a single species.

At the age of one month, and occasionally at 30–36 months, small cells were found to be immunopositive for the NF-L (Mab1615 and the anti-N-terminal NF-L antibody, data not shown). The localization, shape and size of these cells suggest that they are of the glial type. Alternatively, at least in the one month animals, these cells may represent relatively undifferentiated cells which develop into either neurons or glia (followed by a loss of NF expression). However, the occasionally observed reappearance of NF-L-like-IR in this type of cell in animals of 30–36 months makes this hypothesis unlikely. Neurofilaments are considered to be neuron-specific,⁶¹ and NF-like-IR in glial cells, therefore, was unexpected. This finding may either be species specific (and hence a relatively curious finding with no general implications) or may reflect an artifact of antibody binding. However, NF expression in glial cells has previously been reported in embryonic and aged tissue. Galileo and Linser¹⁵ reported the expression of NF-M in glia-like cells in embryonic cell cultures of the optic



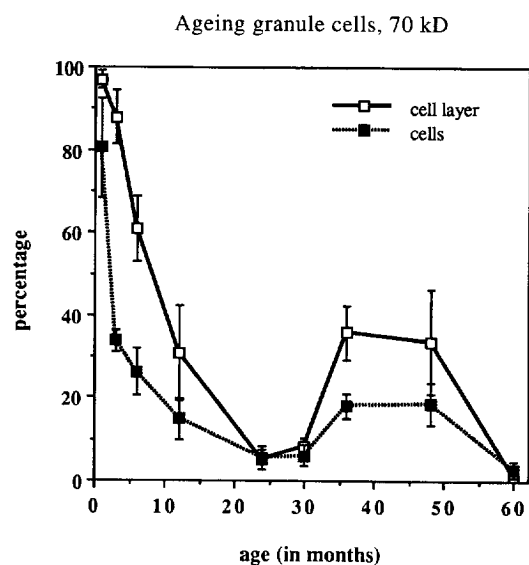


Fig. 5. Two complementary measures were taken to quantify the granular NF-L staining during ageing. The percent of immunoreactive granule cells (closed squares and dashed line) and the percent of the granule cell layer endowed by labelled cells (open squares and solid line) transiently changed over age. Each value represents the mean and S.E.M. See Table 2 for the number of animals studied/age group.

tectum, and fine processes of presumed microglial cells and (occasionally) astrocytes stained for NF-M in the ageing human hippocampus.⁴⁹ Furthermore, Geisert *et al.*¹⁸ and, more recently, Lin and Matesic³⁵ demonstrated that other neuron-specific proteins (microtubule-associated protein 2 and neuron-specific enolase) can be expressed in astrocytes. Nevertheless, since these previous observations of NF-IR in glia were obtained with relatively non-specific antibodies to neurofilaments applied to pathologic tissue, and although glial staining in the present study was found with two antibodies raised against NF-L, we prefer to refer to "NF-L-like" immunoreactivity in astrocytes. Taken together, however, it seems that glial cells, in addition to a large variety of transmitter receptors and signal transduction proteins,^{2,63} are capable of producing several so called neuron-specific proteins, possibly including neurofilaments.

Ageing-related changes in neurofilament immunoreactivity

It is well known that antibody binding to neurofilaments can depend on the phosphorylation state of the neurofilaments. Alterations in NF-IR observed during ageing may be due to changes in the degree of phosphorylation if the epitope of the antibody used is near or at a phosphorylation site.⁵⁶ Employing antibodies dependent on the phosphorylation state may reveal alterations not observed for those independent of the phosphorylation state. Therefore, knowledge of the characteristics of the antibodies used is a prerequisite for proper interpretation of the data obtained. The antibodies N52, NN18, and Mab1615 were employed to study age-related changes. The phosphorylation experiments indicate that N52 and NN18 are phosphorylation-independent antibodies, whereas Mab1615 binds only to the phosphorylated form of NF-L in the rabbit brain. Shaw *et al.*⁵² also found that N52-IR is not influenced by enzymatically dephosphorylation of NF-H. The results with NN18, however, are less consistent. NN18 has been reported to bind to NF-M independently of the phosphorylation state in *E. coli*,⁵² whereas this antibody did not recognize phosphorylated epitopes in the immunogenic lysine-serine-proline repeats of NF-M in enteric neurons.¹³ To our knowledge, there are no previous reports regarding the relation of Mab1615-IR to the phosphorylation state of NF-L.

The number of NF-H-positive interneurons in the stratum oriens/pyramidale declined with age. In contrast, the number of NF-L-positive interneurons, which were indistinguishable from the NF-H group regarding cell type, orientation and localization in the stratum oriens/pyramidale, did not reduce with age. This decline in number of immunoreactive cells cannot be explained by a methodological flaw due to the increased size of the hippocampus in older animals. The length of the sampled stratum oriens (at the level of 56–60 according to the rabbit brain atlas of Shek *et al.*⁵³) was estimated to increase 12% in 60 months as compared to one-month-old animals, whereas the number of labelled interneurons decreased by almost 40%. The reductions in the NF-H-positive interneurons were found at an early stage (around three to six months) and a late stage (around 30–36 months). The first decline most likely reflects a maturation effect, whereas the latter may be interpreted as an

Fig. 4. The number of granule cells immunoreactive for NF-L gradually decreases during ageing (the age is indicated at the upper right corner). Immunopositive granule cells are often found in small clusters (large arrows in D, F and G) at ages over six months. At the age of 24 (E) and 30 months (data not shown), NF-L-IR is diminished in the granular cell layer. However, a transient reappearance of NF-L-IR granule cells occurs at the age of 36–48 months (F and G, respectively). At the age of 60 months (H), all granule cells are nearly immunonegative. Interneurons of the hilar region (small, bold arrows) and glia cells (small arrows) are immunopositive in young rabbits of one month, and occasionally in animals of 30–36 months. Mol, stratum moleculare; Gr, stratum granulare; Hil, hilar region. Scale bars in A–H=25 μ m.

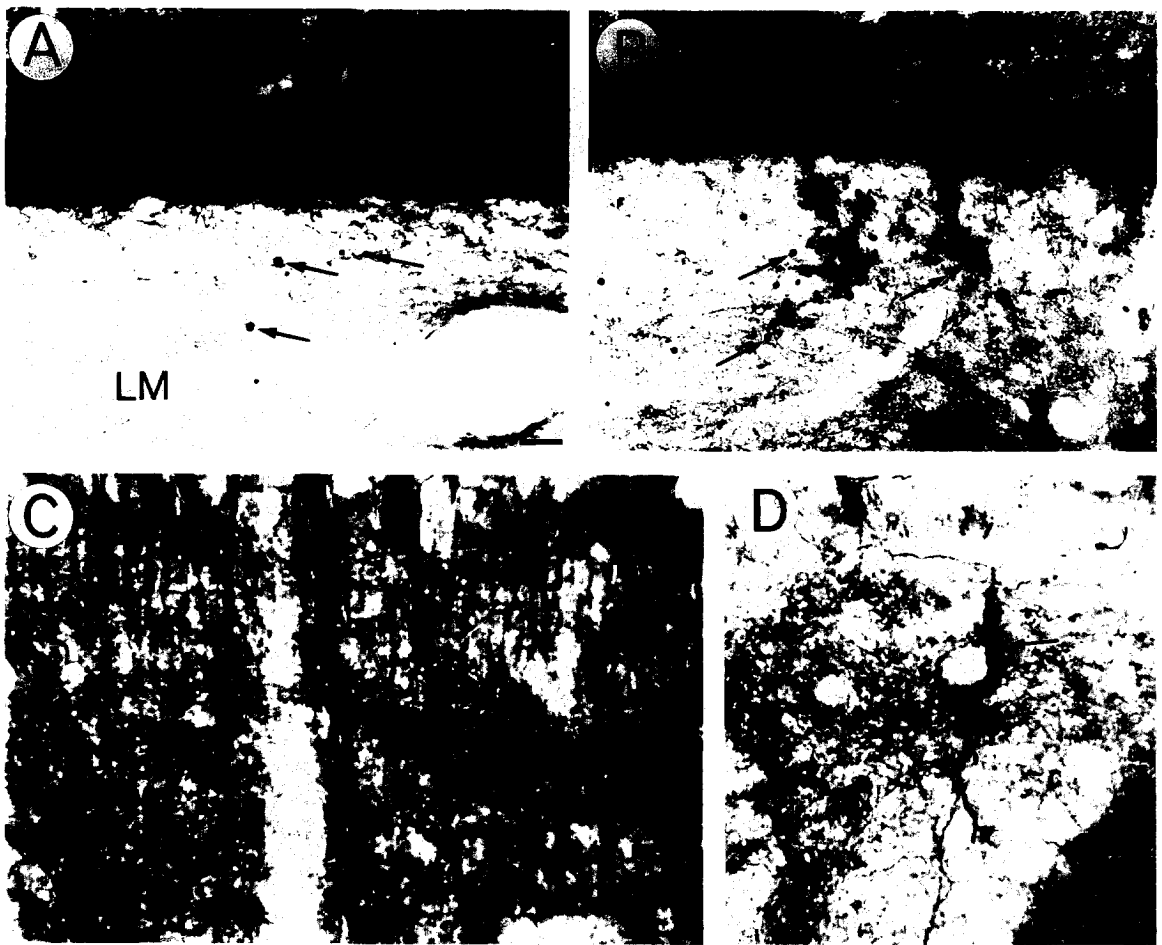


Fig. 6. Axonal aberrations are observed for all NF-subunits (all aberrations shown in this figure are stained for NF-H), with increasing incidence and maximal size in the ageing animals. The aberrations are most frequently found in the stratum lacunosum moleculare, although relatively sparse at the age of 24 months (arrows in A), but extensively at the age of 60 months (arrows in B). However, the axonal aberrations can be found throughout the hippocampal formation, for example in the stratum radiatum (arrows in C) and the stratum moleculare of the dentate gyrus (arrows in D) of a 60-month-old animal. Rad, stratum radiatum; LM, stratum lacunosum moleculare. Scale bars in A and B=50 μ m, in C and D=13 μ m.

ageing effect. De Jong *et al.*¹⁰ found a somewhat similar profile for calbindin-stained interneurons in the hippocampus proper using brain sections from the same animals, whereas no age-related change was seen in the number of interneurons immunoreactive for the calcium binding protein, parvalbumin. Taken together, the observations suggest that calbindin and NF-H may be co-expressed in the same neurons, which seem to be more affected by maturation and ageing than other groups of hippocampal interneurons.

Like in the rabbit, no changes were observed for NF-H in ageing pyramidal cells of rats and mice. Homogenates of the hippocampus revealed no changes in NF-H detected by radioimmunoassay between three and 18-month-old mice, or three- and 24-month-old rats.⁴⁵ The increase in NF-L staining in the pyramidal cells in the present study was moderate, and only seen in the CA1 at the ages of 48 and 60 months.

Dramatic and complex changes in NF-L-IR were found in ageing granule cells, in contrast to the pyramidal cells. A gradual decrease in NF-L staining intensity was found in these cells of animals from one to 24–30 months until it was no longer detectable; the immunoreactivity transiently reappeared, most notably in granule cells lining the hilus, at the age of 36–48 months. Reappearance of NF-L-IR was also observed in dentate hilar interneurons and glial cells, although to a lesser extent. It is known that outbred strains, such as the New Zealand White rabbits used in this study, may have increased inter-animal variability. However, it seems unlikely that the observed reappearance of NF-L-IR is due to genetic diversity instead of the biology of ageing. The mean of the population in an outbred strain is not expected to be affected, and the significant difference between either the 36 or 48-month-old animals and the 24–30-month-old animals indicates that this difference may reflect a relevant phenomenon.

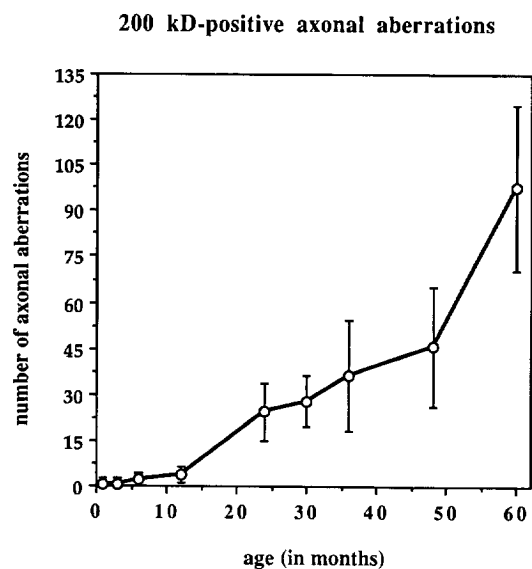


Fig. 7. Age-related increase in the number of NF-H-positive aberrations/mm² in the hippocampal formation. Bars represent S.E.M. See Table 2 for the number of animals/age group.

Furthermore, the simultaneous reappearance of NF-L-like-IR in astrocytes and dentate hilar interneurons suggests that a physiological process underlies the alteration. Several cellular mechanisms may account for the reappearance of NF-L-IR in the granule cells. The NF-L up-regulation most likely indicates an increase in the amount of phosphorylated NF-L which is present in the DG. This could be caused by a shift in the existing pool of NF-L from the dephosphorylated to the phosphorylated state, by enhanced synthesis of NF-L which is subsequently phosphorylated, or by accumulation of phosphorylated NF-L in the cell soma as the result of diminished axonal transport due to degeneration of the granule cells.

Nakamura *et al.*⁴² showed a transient expression of NF-L in CA1 pyramidal cells four days after the induction of ischemia, and considered these pyramidal cells (in which NF-L accumulated in the soma due to a disturbance in axonal transport) to be degenerating. Degeneration of the Mab1615-positive granule cells seems unlikely, since no apparent age-related alterations were observed in the NF-L content of the mossy fibres which arise from the granule cells, and Cresyl Violet staining showed no obvious evidence for dentate granule cell loss. Plastic responses in hippocampal granule cells in NF-IR not only occurred during ageing. Siegel *et al.*⁵⁴ reported that social deprivation increased expression of neurofilaments two-fold in hippocampal granule cells of prepubescent rhesus monkeys using an antibody against NF-M/NF-H. This increase was not observed in other parts of the hippocampus. Their results and ours suggest that NF-regulation in the DG is more plastic than in the hippocampus proper.

Axonal aberrations were observed for NF-M, NF-H, and NF-L if immunostaining was done with NR4, but not with Mab1615. Apparently, the axonal aberrations contain non-phosphorylated NF-L, which is not recognized by Mab1615 (in contrast to NR4⁵²). To form neurofilament triplets, NF-H and NF-M need to be phosphorylated at certain amino acid residues (which predominantly occurs in axons^{31,56}), whereas NF-L, like vimentin-filament, needs to be non-phosphorylated at certain amino acid residues.^{24,26,43} The axonal aberrations immunoreactive for NN18, N52, and NR4, therefore, most likely represent abnormal accumulations of neurofilaments assembled into triplets. The transmitter nature and localization of the cells of origin of the malformed axons remains uncertain. No ChAT-positive aberrations were encountered in the hippocampus, and only some somatostatin-positive aberrations were found. The malformed axons may arise from neurons which use somatostatin as a neurotransmitter, but based on the location, size and number of the somatostatin-positive aberrations, it is likely that the NF-regulation in other transmitter systems are more severely impaired, for example the glutamatergic commissural system.

Different protein kinases and phosphatases are known to regulate the assembly, transport and disposition of neurofilaments (see Ref. 44 for review). It is suggested that NF-phosphorylation increases with age,⁴ and enhanced cytosolic Ca²⁺ concentrations in the ageing hippocampus, either occurring by increased influx of Ca²⁺ or by consistently elevated intracellular Ca²⁺ levels^{12,29,30,47} may account for the increase in phosphorylation by activating Ca²⁺-dependent kinases. The observation by Vega *et al.*⁶⁶ that age-related changes in NF-IR in rat cerebellar cortex could be countered with a dihydropyridine-type Ca²⁺ channel blocker is in agreement with this line of reasoning. The ageing-related increase in NF-L-IR in CA1 pyramidal cells and DG granule cells observed in the present study is therefore consistent with a postulated enhancement in the level of Ca²⁺-mediated phosphorylation of NF-L.

Ageing-related changes in neurofilament immunoreactivity and learning impairments

Little information is available about the role of neurofilaments in learning and memory, but it is interesting to speculate how ageing-related alterations in NF-IR may contribute to age-associated learning impairments. The hippocampally-dependent trace eyeblink conditioning task has been used to determine the age of onset and the severity of age-associated impairments in acquisition in albino rabbits up to an age of 36 months.⁶⁰ The proportion of animals failing to reach an 80% criterion continued to increase with age, being approximately 0, 30, 40, and 60% at the age of 12, 24, 30, and 36 months, respectively. Disruption of neurofilaments interferes

with slow axonal transport, and the occurrence of the observed NF-aberrations would disrupt neuronal function and signal transduction in the ageing rabbit hippocampus. Degeneration of the axon distal to the neurofilament accumulations and subsequent loss of synaptic contacts may eventually take place. The increase in number of axonal aberrations found in the hippocampal formation with advancing age correlates behaviourally with the gradual increase in percentage of animals that fail to reach criterion. At the age of 12, 24, 30, and 36 months, the number of aberrations per mm² increased from 3.9, 24.3, 28.0, to 36.4, respectively, whereas the proportion of animals at these ages failing to reach behavioural criterion was approximately 0, 30, 40, and 60%, as mentioned above. The increase in axonal aberrations was paralleled by a somewhat faster decline of NF-L-IR in dentate granule neurons. This reduction actually occurred somewhat more quickly than the increase in axonal aberrations, as it reached minimum levels by 24 months. It is possible that the increased axonal aberrations combined with the reduced NF-L-IR contribute to the learning deficits which first become evident at 24 months.

The rate of acquisition of rabbits reaching the 80% criterion decreased with age as well, but, surprisingly, those 36 month animals that learned the task did so as quickly as 30 month animals. The up-regulation of NF-L in the DG, which was first seen at the age of 36 months, may (in part) underlie the observed plateau in the rate of acquisition. Neurochemical changes in the DG were induced by trace eyeblink conditioning,⁶⁴ demonstrating that the DG plays a role in this learning task. The up-regulated NF-L-IR in the DG of 36-month-old animals, to the point of resembling the degree of NF-L-IR seen in young animals, may enable a proportion of the older animals to have a relatively high rate of acquisition.

The present results predict that animals of 48 months of age would have a rate of acquisition similar to animals of 30 and 36 months of age, while 60 month animals would show a further decrease in

the rate of acquisition. The proportion of animals failing to reach an 80% criterion, however, will continue to increase with advancing age. Future experiments will further elucidate the role of neurofilaments in learning and memory processes, especially in ageing brain.

CONCLUSION

NF-IR in the rabbit hippocampus dominated in CA3/CA4 pyramidal cells, and in various fibre systems (alveus, mossy fibres, fimbria and fibres scattered throughout the hippocampal formation), whereas granule cells, interneurons, and CA1/CA2 pyramidal cells usually stained less intensely. Besides neuronal immunoreactivity, NF-L-like-IR was found in glial cells at the ages of one month in all hippocampal fields and, occasionally, in the DG of 30–36-month-old animals. Age-related loss of NF-IR was observed for NF-H in interneurons of the stratum oriens/pyramidale and (transiently) for NF-L in the granule cells. In contrast, a slight increase for NF-L was observed in CA1 pyramidal cells. Axonal aberrations were observed for all neurofilament subunits, increasing in maximal size and number with age, suggesting malformation of excessive NF-triplets in distal axons. These changes, combined with the decline of NF-L-IR in granule cells, may (in part) underlie age-associated learning impairments. Our results show that the DG is more plastic in ageing-related regulation of NF expression than the hippocampus proper, and that the rabbit hippocampus may be an interesting model to investigate the functional role and some less well documented features of neurofilaments in the ageing brain.

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